REMARKS

Claims 8-19 are pending and under examination in the above-referenced application. No claims have been amended, added or cancelled herein. Thus, claims 8-19 are still pending and under examination.

October 4, 2011 Examiner's Interview

Applicants wish to thank Examiner Channavajjala for her time and consideration during the October 4, 2011 telephonic interview with Alan J. Morrison, Esq., applicants' undersigned attorney. During the interview, the Examiner and Morrison discussed the outstanding novelty and obviousness rejections. It was agreed that that withdrawal of these rejections would be favorably considered were applicants to submit evidence showing that (i) spider veins and varicose veins are distinct disorders having distinct treatments, and/or (ii) the acid form of a therapeutic agent typically behaves differently than its salt form regarding skin penetration. As set forth in detail below, applicants provide such evidence, and respectfully request that the outstanding rejections be withdrawn.

Rejection Under 35 U.S.C. §102(b)

The Examiner rejected claims 8-19 under 35 U.S.C. §102(b) as allegedly anticipated by Znaiden, et al. (U.S. Patent No. 5,268,176 ("Znaiden ('176")")), as evidenced by James, et al. (Radiographic, 1999, pages 1093-1099 ("James")).

In response, applicants respectfully traverse.

Claim 1 provides a method for treating soft tissue calcifications. The method comprises topically administering a composition comprising an effective amount of myo-inositol hexaphosphate or a pharmaceutically acceptable salt thereof (i.e., phytate). The phytate can then be absorbed by the skin, passing into the bloodstream and acting where the soft tissue

calcification is generated. Claim 14 provides a corresponding method for preventing soft tissue calcifications.

Znaiden ('176) fails to teach all elements of the claimed methods, inherently or otherwise.

Specifically, Znaiden ('176) teaches a method for treating spider veins by topically administering phytic acid. For the Examiner's rejection to have merit, the method of Znaiden ('176) would *necessarily* have to result in the treatment of soft tissue calcification via topical phytate administration. Applicants maintain that the method of Znaiden ('176) results in no such treatment. A brief review of James and the relevant art makes this point clear.

The Examiner cites James as evidence that Znaiden ('176) inherently anticipates the claimed method. That is, according to the Examiner, James teaches, at least in part, that (i) soft tissue calcification and varicose veins necessarily coexist, and (ii) by implication, spider veins and varicose veins are equivalent as to etiology and treatment. Applicants stress that both of these assumptions are incorrect.

First, the James reference teaches nothing more than the medical fate of a single 15-year-old patient afflicted with Klippel-Trénaunay syndrome, as reflected in the reference's title "Pediatric Case of the Day." In relevant part, this patient was afflicted with varicose veins and calcified phleboliths, among other disorders. Based on the singular example in James, one cannot reasonably conclude that a subject having varicose veins is necessarily afflicted with soft tissue calcification. Nothing in James suggests otherwise.

Second, varicose veins and spider veins are different disorders having different treatments. In support of this point, applicants cite, for example, the website for the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/mesh/68014652). This site defines spider veins (i.e., telangiectasia) as a "[p]ermanent dilation of preexisting blood vessels

[capillaries, arterioles and venules] creating small focal red lesions, most commonly in the skin or mucous membranes. It is characterized by the prominence of skin blood vessels, such as vascular spiders." This site also defines varicose veins as "[e]nlarged and tortuous" veins. Applicants further cite the website http://www.spiderveinsonlegs.org/treatment-for-spider-veins/, which states that "[t]reatment for spider veins is much different than the vein stripping for varicose veins; it is much simpler and less invasive than surgery."

Since James fails to establish that varicose veins and spider veins are equivalent, or that tissue calcification necessarily afflicts every subject having varicose veins, this reference does not show that Znaiden ('176) inherently teaches the claimed methods.

In view of the above, applicants maintain that the claimed methods are novel over Znaiden ('176) in view of James, and that therefore, the pending claims satisfy the requirements of 35 U.S.C. §102(b).

Rejection Under 35 U.S.C. §103

The Examiner rejected claims 8-19 under 35 U.S.C. §103(a) as allegedly obvious over either of Znaiden ('176) or Znaiden (U.S. Patent No. 5,552,148 ("Znaiden ('148)")), in view of Horrobin, et al. (U.S. Patent No. 5,516,801 ("Horrobin")) and Kamiya (U.S. Publication No. 2003/0119910 ("Kamiya")), as evidenced by James.

In response, applicants respectfully traverse.

Znaiden ('176) and James are discussed above. Znaiden ('148) is cited as teaching phytic acid as a cosmetic additive. Kamiya and Horrobin are discussed in detail in applicants' April 1, 2009 Response, and applicants have not reproduced those discussions here.

In essence, the Examiner again asserts that James establishes a correlation between the presence of varicose veins and tissue calcification. As applicants have shown, this correlation

does not exist. Likewise, applicants have shown that varicose veins and the spider veins treated in Znaiden ('176) are different disorders having different treatments.

Indeed, as discussed during the October 4 interview, applicants maintain that Znaiden ('176) *teaches against* the claimed method. Specifically, at col. 3, l. 16-24, Znaiden ('176) states that "phytic acid ... has twelve distinct pK values equally distributed between pH 1.1 and pH 12.0. Once a highly polar molecule reaches viable tissue, it will be repulsed by cell membrane phospholipids and *remain* in the intercellular space. Instead of being lost through dissipation, the molecules remain *sequestered* and form a *stable depot,* which creates a high osmotic gradient necessary for the collapse of an offending vessel." (emphasis added) That is, at physiological pH, phytic acid becomes polar through the loss of protons. Based on Znaiden ('176), one of ordinary skill would have expected the claimed methods to *fail* due to phytate sequestration in the intercellular space. One skilled in the art would *not* have reasonably expected phytate to enter the body systemically to treat soft tissue calcification.

Applicants' position is also based on the general notion that it is difficult for the salt form (i.e., polar form) of an acid to penetrate the skin's stratum corneum. In support of this position, applicants cite M. Prausnitz, et al. (Proc. Natl. Acad. Sci. USA, Vol. 90, pp. 10504-10508, November 1993), at p. 10508, second paragraph, which states that "[b]ecause of the stratum corneum's overall hydrophobic character and net negative charge, transdermal transport of negatively charged hydrophilic molecules is *especially challenging*." (emphasis added) Applicants also cite B.W. Barry (European Journal of Pharmaceutical Studies 14 (2001) 101-114), at p. 103, under 4.1.3, which states that "[c]harged molecules do *not* readily penetrate [the] stratum corneum." (emphasis added)

In view of the adverse teaching of Znaiden ('176), especially in light of the information above, applicants maintain that one of ordinary skill would not have reasonably expected the

claimed methods to succeed. Horrobin (teaching soft tissue calcification generally) and Kamiya (teaching the treatment of Klotho protein-related disorders) fail to overcome the shortcomings of Znaiden (176) and Znaiden ('148) as evidenced by James. Specifically as to Kamiya, this reference recites "phytic acid" in a lists of compounds, and separately recites "ectopic calcification" in a list of disorders. Kamiya does not combine these two notions to constitute a specific treatment method, and does not otherwise suggest a treatment approach that would cure the shortcomings of the Znaiden references.

In view of the above, applicants maintain that the claimed methods are not obvious over Znaiden ('176) or Znaiden '148, in view of Horrobin and Kamiya, as evidenced by James. Thus, applicants maintain that the pending claims satisfy the requirements of 35 U.S.C. §103.

It is believed that no fees or charges (other than the one-month extension fee) are required at this time in connection with the present application. However, if any additional fees or charges are required, they may be charged to our Patent and Trademark Office Deposit Account No. 503111.

Respectfully submitted,
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Electroporation of mammalian skin: A mechanism to enhance transdermal drug delivery

(stratum corneum/lipid bilayer/electropermeabilization/tissue/iontophoresis)

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ABSTRACT **Mammalian skin owes its remarkable barrier function to its outermost and dead layer, the stratum corneum. Transdermal transport through this region occurs predominantly through intercellular lipids, organized largely in bilayers. Electroporation is the creation of aqueous pores in lipid bilayers by the application of a short (microseconds to milliseconds) electric pulse. Our measurements suggest that electroporation occurs in the intercellular lipid bilayers of the stratum corneum by a mechanism involving transient structural changes. Flux increases up to 4 orders of magnitude were observed with human skin *in vitro* for three polar molecules having charges between −1 and −4 and molecular weights up to slightly more than 1000. Similar flux increases were observed *in vivo* with animal skin. These results may have significance for drug delivery and other medical applications.**

Transdermal drug delivery offers a number of potential advantages over conventional methods, such as pills and injections: (*i*) no degradation due to stomach, intestine, or first pass of the liver, (*ii*) probable improved patient compliance because of a user-friendly method, and (*iii*) potential for steady or time-varying controlled delivery (1–4). Nevertheless, very few drugs can be administered transdermally at therapeutic levels, due to the low permeability and lipophilic nature of human skin. As a result, fewer than 10 drugs are now clinically administered transdermally. However, the market for these drugs exceeds one billion dollars in the United States alone, indicating the importance of this delivery method. Therefore, significant enhancement of transdermal drug delivery has the potential for major impact on medicine.

A number of approaches have been taken to increase transdermal transport (2–4). Most common is the addition of chemical enhancers, compounds which are believed to increase the partitioning of drugs into the skin. Another approach is chemical modification of a drug into a "prodrug," which penetrates the skin well but is subsequently converted by epidermal enzymes into the original pharmacologically active drug. Application of ultrasound has been used as well to increase transdermal flux and to reduce transport lag times. Yet another approach is iontophoresis, the movement of drugs across the skin by an electric field. Mechanistically similar to electrophoresis, iontophoresis is believed to act primarily by moving charged species across the skin by an electrical force.

The barrier properties of skin are attributed primarily to the stratum corneum, the skin's outer layer. The stratum corneum is a dead tissue composed of flattened cells filled with crosslinked keratin and an extracellular matrix made up of lipids arranged largely in bilayers (5, 6). Unlike the unilamellar phospholipid bilayers of cell membranes, these multilamellar, extracellular bilayers contain no phospholipids, being composed primarily of ceramides, cholesterol, and fatty acids (1–3). Intercellular pathways are generally the most important routes for transdermal transport (1–3). Therefore, permeabilization of the lipid bilayers filling these intercellular pathways would be expected to increase transdermal transport.

Electroporation is a method of reversibly permeabilizing lipid bilayers, involving the creation of transient aqueous pores by the application of an electric pulse (7, 8). Dramatically reduced electrical resistance and extensive transport of molecules, including macromolecules, are generally associated with electroporation of lipid bilayers, including membranes of artificial planar and spherical systems, as well as those of living cells. Electric field exposures causing electroporation typically generate transmembrane potentials of $\approx$1 V and last 10 μsec to 10 msec. Electroporation of isolated single cells is well established, but electroporation of cells that are part of an intact tissue has received little attention (9–12). To our knowledge, electroporation of multilamellar or non-phospholipid systems has not been previously demonstrated.

In this study, we examine the possibility of electroporating the multilamellar, non-phospholipid, intercellular lipid bilayers of the stratum corneum as a mechanism to enhance transdermal drug delivery. Although both electroporation and iontophoresis involve electric fields, the two approaches are fundamentally different. While iontophoresis acts primarily on the drug, involving skin structural changes as a secondary effect (2, 3), electroporation is expected to act directly on the skin, making transient changes in tissue permeability. Because electroporation of cells has been shown to increase transmembrane fluxes dramatically and reversibly, electroporation of skin could make possible the transdermal delivery of many more drugs at therapeutic levels.

MATERIALS AND METHODS

Materials. Phosphate-buffered saline (PBS) was prepared containing 138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.1 mM KH_2PO_4 (Mallinckrodt) and was adjusted to pH 7.4 by adding NaOH or HCl (Mallinckrodt). Calcein was obtained from Sigma and Molecular Probes. Lucifer yellow and erythrosin-5-iodoacetamide were obtained from Molecular Probes. To make the sulfur-alkylated erythrosin derivative, erythrosin-5-iodoacetamide was incubated with excess 6-mercapto-1-hexanol in PBS at 25°C for >12 hr.

Skin Preparation. By use of established methods for skin sample preparation, full-thickness excised cadaver skin was obtained within 48 hr after death and stored at 4°C and 95% humidity for up to 1 week (2, 3). Full-thickness samples were prepared by gently scraping off subcutaneous fat. Epidermis samples were heat separated by submerging full-thickness

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skin in 60°C water for 2 min and gently removing the epidermis (13). All samples were stored at 4°C and 95% humidity for <3 weeks. Tissue was obtained from four sources (three local hospitals and the National Disease Research Interchange) to minimize any artifacts of tissue acquisition. Tissue was generally from the abdomen, removed just lateral to the midline, although tissue from the breast, back, and thigh have been used as well.

Because the primary barrier to transport is the stratum corneum (the upper 10–20 μm of the epidermis), the use of epidermis rather than full-thickness skin is a well-established model for transdermal drug delivery (2, 3). In the literature, transdermal drug delivery is commonly understood to mean transport of drugs across the skin (not just the dermis) (1–4). When systemic delivery is desired, a drug must traverse the stratum corneum, the viable epidermis, and some fraction of the dermis before entering blood vessels of the systemic circulation. Since capillaries exist near the dermal/epidermal junction, drugs can enter the systemic circulation without crossing the whole dermis (1–3). Thus, transport across full-thickness skin misrepresents the actual transport pathway. For these reasons, following established practice (2, 3), we have performed the majority of our studies with human epidermis and have established agreement with select results from full-thickness human skin.

***In Vitro* Methods.** Prepared skin samples were loaded into side-by-side permeation chambers (14), exposed to well-stirred PBS, and allowed to hydrate fully (12–18 hr, 4°C). The temperature was raised to 37°C and fresh PBS was added, with 1 mM fluorescent compound (calcein, Lucifer yellow, or erythrosin derivative) on the outer, stratum corneum side. After a steady-state flux was established (within a few hours), electric pulsing was applied with Ag/AgCl electrodes (≈2 cm from skin) (In Vivo Metrics, Healdsburg, CA). An exponential-decay pulse (τ = 1.0–1.3 msec; Gene-Pulser; Bio-Rad) was applied every 5 sec for 1 hr, with the negative electrode on the stratum corneum side ("forward" pulsing), except for "reverse" pulsing, where the positive electrode was on the stratum corneum side. Iontophoresis (continuous dc voltage) was also used for 1 hr, with the negative electrode on the stratum corneum side. The receptor compartment was sampled periodically by emptying its contents and replacing it with fresh PBS. Analysis by calibrated spectrofluorimetry (Fluorolog-2, model F112AI; Spex Industries, Metuchen, NJ) allowed measurement of fluorescent compound concentrations in the receptor compartment and, thereby, calculation of transdermal fluxes.

Reported voltages are transdermal values, determined at >1 μsec after the onset of the pulse. During a pulse, the apparent resistance of the chamber, without skin (but including electrodes, saline, and interfacial resistances), was 480 Ω, independent of the pulse voltage. The apparent resistance of the chamber with skin varied from 900 Ω during lower-voltage pulses (≈50 V across skin) to 600 Ω during higher-voltage pulses (≈500 V across skin), meaning that only 20–50% of the applied voltage appeared across the skin. Transdermal voltages were determined by calculating the ratio of the apparent skin resistance to the apparent total chamber (with skin) resistance. This ratio is equal to the ratio of the transdermal voltage to the voltage across the whole chamber (with skin). By application of a voltage pulse and measurement of the resulting current, apparent resistances were calculated by dividing the applied voltage by the measured current.

Post-pulse skin electrical characterization was performed with a four-electrode impedance-measurement system. A current step (2 μA/cm²) was applied and the resulting transdermal voltage was measured. By using a Fourier transform, skin impedance was calculated over a range of frequencies (1–1000 Hz) by dividing the measured transdermal voltage by the applied current.

***In Vivo* Methods.** For *in vivo* studies, reservoirs (≈4 ml, 2.8 cm²) with Ag/AgCl electrodes (≈1 cm from skin) were attached to gently pinched skin from the caudal dorsal region of anesthetized (ketamine, 75 mg/kg, and xylazine, 10 mg/kg, with additional one-third doses given every 30–45 min) CD hairless rats (Charles River Breeding Laboratories) (12); animal care was in accordance with institutional guidelines. Hairless rodents are commonly used as *in vivo* models for transdermal studies (2, 3). Both reservoirs were filled with PBS; the negative electrode side contained 10 mM calcein. Pulses were applied as described above for 1 hr.

Plasma calcein concentration measurements were made 30–60 min after pulsing. Blood samples were taken from the lateral tail vein, transferred into a serum separator tube (Microtainer, Becton Dickinson), and spun at 1000 × *g* for 5 min to isolate the plasma for analysis by calibrated spectrofluorimetry. The appropriate volume of distribution of calcein within the rat was determined by measuring plasma concentrations over time following intravenous and subcutaneous injections of known amounts of calcein. Maximum plasma concentrations were measured 30–60 min after injection, suggesting that significant metabolism or elimination did not occur over that period (15, 16). The volume of distribution determined from these measurements was 20% of total rat volume (17), which is equal to the volume of the extracellular aqueous compartment (18). Given the very hydrophilic nature of calcein (19), distribution throughout all extracellular aqueous regions is a reasonable assumption.

RESULTS AND DISCUSSION

To determine whether electroporation of the stratum corneum was possible, we subjected human cadaver epidermis under physiological conditions to electric pulses which cause electroporation in other systems. Quantitative measurements of transdermal molecular flux, supported by electrical measurements, are consistent with three unique characteristics of electroporation (7, 8): (*i*) large increases in molecular flux and ionic conductance, (*ii*) reversibility over a range of voltages, and (*iii*) changes in barrier membrane structure.

First, transdermal fluxes of calcein (623 Da, −4 charge), a moderate-sized, highly polar molecule which does not normally cross skin in detectable quantities, were measured during application of low-duty-cycle electric-field pulses. Fig. 1 shows average transdermal fluxes of calcein before, during, and after pulsing at representative voltages. Fluxes before pulsing were below the detection limit (imposed by background fluorescence), whereas fluxes during pulsing were up to 4 orders of magnitude above this limit. Fig. 2 shows that flux increased nonlinearly with increasing pulse voltage, where flux increased strongly with increasing voltage below ≈100 V and increased weakly with increasing voltage at higher voltages. Supporting electrical measurements also showed increases in skin conductance of 1–3 orders of magnitude. Electrical changes were evident during the pulse and immediately (≥10 msec) after. This is consistent with changes caused by electroporation, the onset of which is believed to occur on the microsecond scale (20–22).

Second, reversibility was assessed. Following electrical pulsing for 1 hr, transdermal fluxes generally decreased by about 90% within 30 min and by >99% within 1 or 2 hr (Fig. 1), indicating significant reversibility. Electrical conductance measurements also showed recovery, either complete or to within a factor of 3 of pre-pulse values. However, elevated post-pulsing fluxes could be caused not only by irreversible alterations of skin structure but also by the efflux of calcein "loaded" into the skin during high fluxes during pulsing (23, 24).

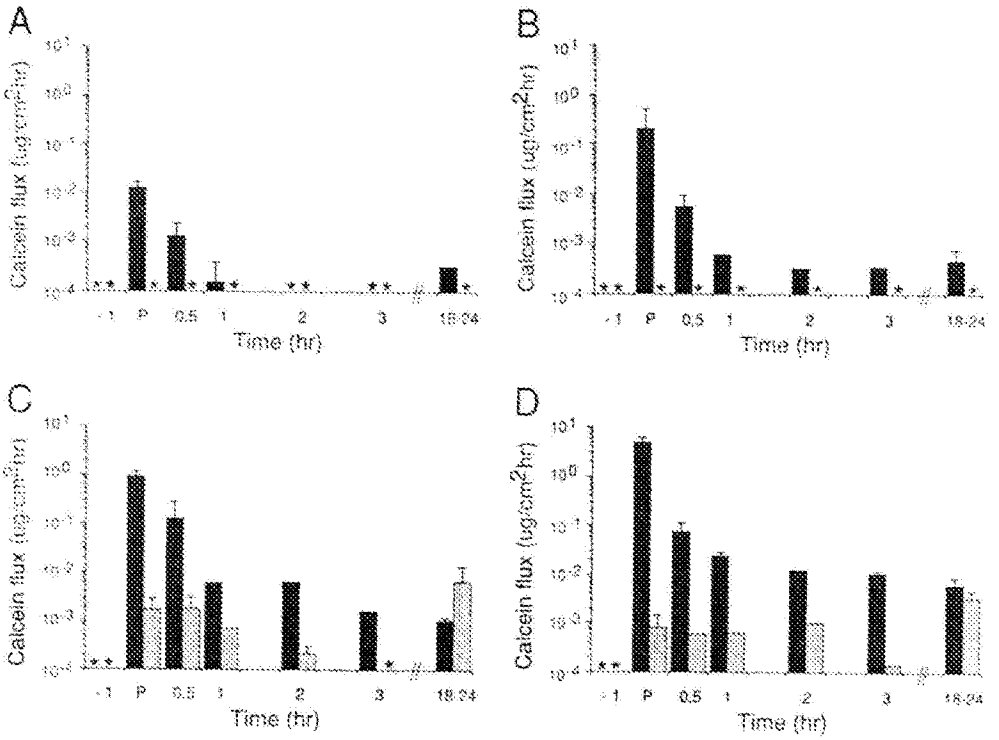

FIG. 1. Transdermal fluxes of calcein (623 Da, −4 charge) before, during, and after "forward" pulsing (solid bars) or "reverse" pulsing (stippled bars) at 55 V (*A*), 90 V (*B*), 165 V (*C*), and 300 V (*D*). Flux increases up to 4 orders of magnitude are observed under "forward" pulsing conditions (see text). These increases are at least partially reversible. "Reverse" pulsing facilitates independent assessment of changes in skin permeability due to electroporation (see text), suggesting that skin electroporation may be fully reversible below ≈100 V, under the conditions used. Fluxes are shown 1 hr before pulsing, during pulsing (P), and at times after pulsing. Pulsing was performed for 1 hr (see text). Elevated fluxes at 18–24 hr could be caused by skin deterioration. Each point represents the average of three to seven skin samples, from two to four different subjects. Standard deviation bars are shown. Asterisk indicates a flux below the detection limit, of order 10^{-4} $\mu g/(cm^2 hr)$.

The results of an additional, and possibly better, test of reversibility are also shown in Fig. 1: skin was pulsed with the electrode polarity reversed, leaving the transmembrane voltage magnitude during pulsing the same. However, the electrophoresis associated with the pulse should move calcein away from the skin under these conditions, markedly reducing transdermal transport during pulsing. By measuring fluxes ≈1 hr after reverse-pulsing, long-lived changes in skin permeability can be assessed independently, as summarized in Fig. 2. These data suggest that pulses at or below ≈100 V cause no detectable long-lived changes in skin permeability. However, higher voltage pulses appear to cause lasting changes; these changes do not go away, even after 18–24 hr. Fig. 2 also suggests that a transition region may exist at ≈100 V, below which flux increases as a strong function of voltage and flux increases are reversible, and above which flux increases only weakly with voltage and effects are only partially reversible. The exact mechanism underlying this transition is unclear.

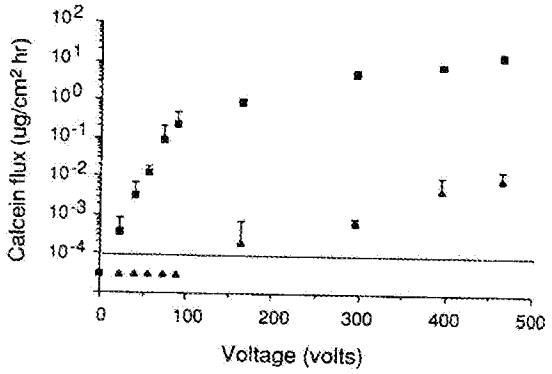

FIG. 2. Transdermal fluxes of calcein due to exposure of human skin to different electrical conditions. Calcein flux during application of forward-polarity pulses (■) and ≈1 hr after pulsing in the reverse direction (see text) (▲). This figure suggests that a transition point may exist at ≈100 V, below which flux increases as a strong function of voltage and flux increases are reversible, and above which flux increases only weakly with voltage and effects are only partially reversible. Each point represents the average of three to seven skin samples, from two to four different subjects. Standard deviation bars are shown. Fluxes below the calcein flux detection limit are indicated below the dashed line.

Third, changes in skin structure cannot be expected to be revealed by microscopy, for reasons discussed below. However, demonstrating that increased fluxes caused by pulsing cannot be explained by electrophoresis alone suggests that changes in skin structure are necessary to explain our results. We therefore compared fluxes caused by low-duty-cycle high-voltage pulsing to fluxes caused by the continuous low-voltage dc current which would provide the same total electrophoretic component if no changes in skin structure occurred. For example, if the skin were unaltered (i.e., same conductance), then continuous application of 0.1 V would transfer the same amount of charge across the skin as the pulsed application of 500 V for 1 msec every 5 sec, making these conditions electrophoretically "equivalent." As seen in Fig. 3, application of continuous voltages caused fluxes 3 orders of magnitude smaller than pulsing under "equivalent" conditions, suggesting that skin structural changes are needed to explain these results.

To assess the occurrence and reversibility of electroporation, we believe that characterization of flux changes, along with companion electrical measurements, is the best approach, since these measures are universally accepted in the

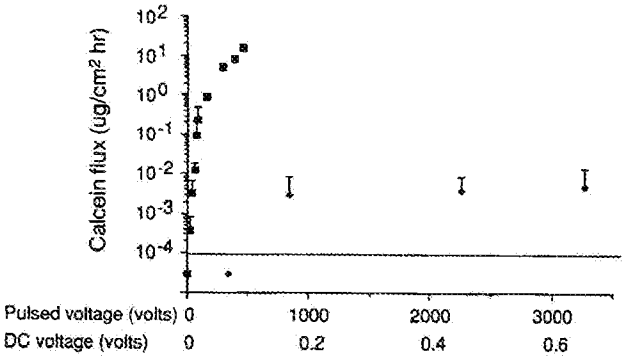

FIG. 3. Transdermal fluxes of calcein during pulsing (■) and during application of dc iontophoresis (♦). Upper axis indicates pulsing voltages electrically "equivalent" to continuous dc voltages on lower axis (see text), suggesting that skin structural changes may be needed to explain the high fluxes caused by electroporation. Each point represents the average of three to seven skin samples, from two to four different subjects. Standard deviation bars are shown. Fluxes below the calcein flux detection limit are indicated below the dashed line.

electroporation literature (7, 8). Upon initial consideration, electron microscopy might also appear to be an appropriate tool for visualizing the pores created by electroporation. However, there currently exist no satisfactory electron micrographs of electropores in any membrane, primarily because electropores are believed to be small (<10 nm), sparse (<0.1% of surface area), and generally short-lived (microseconds to seconds) (7, 8). Thus, it is extremely difficult to visualize electropores by electron microscopy. Moreover, although the name electroporation suggests the creation of physical pores, all that has been concretely established is that a transient high-permeability state is created, characterized by greatly increased permeability and electrical conductivity (7, 8). We therefore did not employ electron microscopy to look for pores in the complex multilaminate structures of the skin, since their existence had not been established in simpler systems.

Enhanced transport of two other polar molecules across the skin was achieved by electroporation: Lucifer yellow (457 Da, −2 charge) and an erythrosin derivative (1025 Da, −1 charge), a small macromolecule, neither of which normally crosses skin at detectable levels. These molecules were selected because they are fluorescent and have different physical properties than calcein. As seen in Fig. 4*A*, pulsing can cause fluxes of both molecules similar to those caused for calcein under the same conditions. This suggests that electroporation-enhanced transport may be broadly applicable to many molecules, possibly including those of higher molecular weights.

We have also observed flux increases due to pulsing of full-thickness human skin, suggesting that artifacts due to epidermis preparation are not significant. However, full-thickness fluxes were delayed (about 1 hr) and were about an order of magnitude lower, probably due to binding or diffusional limitations in the dermis (2–3 mm thick). Moreover, we have observed up to 1000-fold flux increases due to pulsing in frog and hairless rat skin *in vitro* (data not shown).

Finally, electroporation *in vivo* was performed on hairless rats, assessed by measuring serum concentrations of calcein delivered transdermally (Fig. 4*B*). Fluxes in excess of 10 $\mu g/(cm^2 hr)$ were observed at voltages as low as 30 V; these fluxes are at least 2 orders of magnitude greater than controls (Fig. 4*B*). That the *in vivo* fluxes do not increase with voltage suggests that a rate-limiting step other than transport across the stratum corneum exists, perhaps uptake of calcein from a skin depot into the bloodstream. No visible skin damage

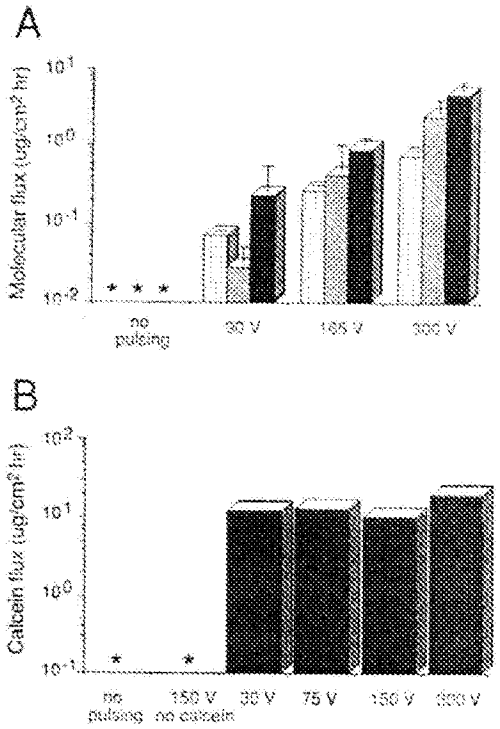

FIG. 4. Transdermal fluxes *in vitro* and *in vivo*. (*A*) Transdermal fluxes of an erythrosin derivative (1025 Da, −1 charge) (stippled bars), Lucifer yellow (457 Da, −2 charge) (hatched bars), and calcein (solid bars) across human skin *in vitro*. This figure demonstrates that electroporation increases the flux of a number of polar molecules having different molecular characteristics. Each point represents the average of three to seven skin samples, from two to four different subjects. Standard deviation bars are shown. Asterisk indicates a flux below the detection limit, of order 10^{-2} $\mu g/(cm^2 hr)$ for the erythrosin derivative and 10^{-3} $\mu g/(cm^2 hr)$ for Lucifer yellow. (*B*) Flux of calcein across hairless rat skin *in vivo*, which suggests that electroporation can increase transdermal flux in a living animal. Each point represents the average result from one or two rats. Asterisk indicates a flux below the detection limit, of order 10^{-1} $\mu g/(cm^2 hr)$.

was observed after pulsing at voltages below 150 V; erythema and edema were evident at higher voltages. Long-term biochemical and pathological studies are needed.

It is well established that the stratum corneum is the primary barrier to transdermal transport (1–4); thus, changes in the stratum corneum probably account for the observed increases in flux due to electroporation. Although it has been applied primarily to living cells, electroporation has also been widely studied in artificial planar bilayer membranes and liposomes (7, 8). Electroporation is a physical process based on electrostatic interactions and thermal fluctuations within fluid membranes; no active transport processes are involved (7, 8). Thus, electroporation could occur in the stratum corneum even though it does not contain living cells.

The stratum corneum has a much higher electrical resistance than other parts of the skin. As a result, an electric field applied to the skin will concentrate in the stratum corneum, resulting in other, viable tissues being exposed to much lower fields. Therefore, under appropriate conditions, an electric field sufficient to cause electroporation could exist in the stratum corneum, while a significantly lower field existed in viable tissues, insufficient to cause electroporation. An implicit targeting mechanism results, where the greatest electric fields are generated where the largest resistivities exist, thereby protecting the already permeable viable parts of the skin and deeper tissues.

It is difficult to state with certainty which electrical conditions will be acceptable for clinical use. Many features,

including pulse voltage/current/energy, pulse length, pulse frequency, duration of total exposure, and electrode size, site, and design, will be important (25). A complete histological examination of the safety of electroporation of skin is beyond the scope of this study. However, that the electrical exposures used were fully reversible over a range of voltages is a strong indication that the procedure is not damaging and may prove to be safe under appropriate conditions. Moreover, there exists a clinical precedent for safely applying electric pulses to skin with voltages up to hundreds of volts and durations up to milliseconds. Such diagnostic and therapeutic applications, which involve stimulation of nerves and may inadvertently cause electroporation of skin, include transcutaneous electrical nerve stimulation, functional electrical stimulation, electromyography, and somatosensory-evoked-potential testing (25, 26).

Because of the stratum corneum's overall hydrophobic character and net negative charge, transdermal transport of negatively charged hydrophilic molecules is especially challenging (2, 3). Calcein, with eight charge sites and a net charge of −4 (19), is therefore considerably more difficult to transport across the skin than many other molecules. Approaches to transdermal flux enhancement involving chemical enhancers have been successful with some lipophilic and moderately polar molecules but are limited in applicability to highly polar and charged molecules (2–4). Iontophoresis has been successfully employed with some polar and charged molecules (2–4). For many drugs, delivery rates in the range of micrograms per square centimeter per hour could be therapeutic, whereas significantly higher rates of delivery may be required for other drugs (2, 3). In general, a 10-fold increase in flux caused by an enhancement method is impressive, and a 100-fold increase is of great interest. Thousandfold increases are rarely found (2, 3). The increases of up to 10,000-fold in flux that are caused by electroporation are therefore potentially very significant and could make possible transdermal delivery of many drugs at therapeutic levels.

Finally, transdermal flux enhancement has been demonstrated with other methods, including chemical, iontophoretic, and ultrasonic (2–4). Because electroporation is mechanistically different, involving temporary alterations of skin structure, it could be used in combination with these or other enhancers. Electroporation may also be useful in other applications involving transport across skin, such as noninvasive sensing for biochemical measurement, gene therapy, and cancer chemotherapy. Together, these results suggest that electroporation of mammalian skin occurs and may be useful as a mechanism to enhance transdermal drug delivery.

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Review

Novel mechanisms and devices to enable successful transdermal drug delivery

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Abstract

Optimisation of drug delivery through human skin is important in modern therapy. This review considers drug–vehicle interactions (drug or prodrug selection, chemical potential control, ion pairs, coacervates and eutectic systems) and the role of vesicles and particles (liposomes, transfersomes, ethosomes, niosomes). We can modify the stratum corneum by hydration and chemical enhancers, or bypass or remove this tissue via microneedles, ablation and follicular delivery. Electrically assisted methods (ultrasound, iontophoresis, electroporation, magnetophoresis, photomechanical waves) show considerable promise. Of particular interest is the synergy between chemical enhancers, ultrasound, iontophoresis and electroporation.

Keywords: Review; Skin enhancement; Iontophoresis; Ultrasound; Liposomes

1. Introduction

Recently, the transdermal route has vied with oral treatment as the most successful innovative research area in drug delivery. In the USA (the most important clinical market), out of 129 drug delivery candidate products under clinical evaluation, 51 are transdermal or dermal systems; 30% of 77 candidate products in preclinical development represent such drug delivery. The worldwide transdermal patch market approaches £2 billion, yet is based on only ten drugs — scopolamine (hyoscine), nitroglycerine, clonidine, estradiol (with and without norethisterone or levonorgestrel), testosterone, fentanyl and nicotine, with a lidocaine patch soon to be marketed. The fundamental reason for such few transdermal drugs is that highly impermeable human skin limits daily drug dosage, delivered from an acceptable sized patch, to about 10 mg. This review deals with ways to raise significantly this low limit for topical systems in general.

2. Drug transport through human skin

Human skin is an effective, selective barrier to chemical permeation (Barry, 1983). In general, the epidermis (specifically, the stratum corneum) provides the major control element — most small water-soluble non-electrolytes diffuse into the systemic circulation a thousand times more rapidly when the horny layer is absent. Thus, to maximise drug flux we usually try to reduce this barrier's hindrance, although sometimes the follicular route may also be important. This review considers how molecules cross intact, healthy skin and considers attempts to circumvent the problems of an almost impermeable barricade exhibiting considerable patient variability.

3. Routes of penetration

At the skin surface, molecules contact cellular debris, microorganisms, sebum and other materials, which negligibly affect permeation. The penetrant has three potential pathways to the viable tissue — through hair follicles with associated sebaceous glands, via sweat ducts, or across continuous stratum corneum between these appendages (Fig. 1).

Fractional appendageal area available for transport is only about 0.1%; this route usually contributes negligibly to steady state drug flux. The pathway may be important for ions and large polar molecules that struggle to cross intact stratum corneum. Appendages may also provide shunts, important at short times prior to steady state

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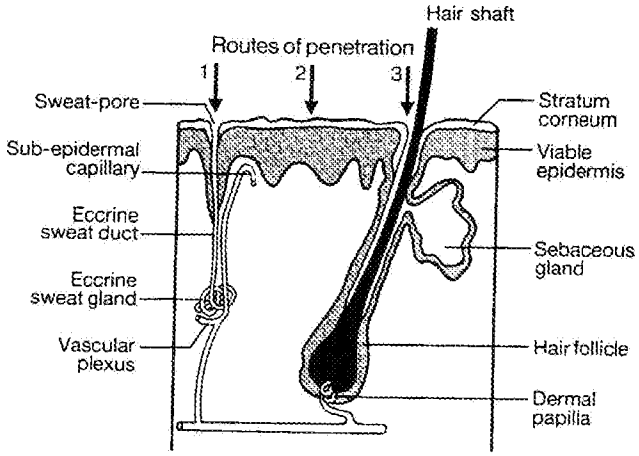

Fig. 1. Simplified diagram of skin structure and macroroutes of drug penetration: (1) via the sweat ducts; (2) across the continuous stratum corneum or (3) through the hair follicles with their associated sebaceous glands.

diffusion. Additionally, polymers and colloidal particles can target the follicle.

The intact stratum corneum thus provides the main barrier; its 'brick and mortar' structure is analogous to a wall (Fig. 2; reviewed in Barry and Williams, 1995). The corneocytes of hydrated keratin comprise the 'bricks', embedded in a 'mortar', composed of multiple lipid bilayers of ceramides, fatty acids, cholesterol and cholesterol esters. These bilayers form regions of semicrystalline, gel and liquid crystals domains. Most molecules penetrate through skin via this intercellular microroute and therefore many enhancing techniques aim to disrupt or bypass its elegant molecular architecture.

Viable layers may metabolise a drug, or activate a prodrug. The dermal papillary layer is so rich in capillaries that most penetrants clear within minutes. Usually, deeper dermal regions do not significantly influence absorption,

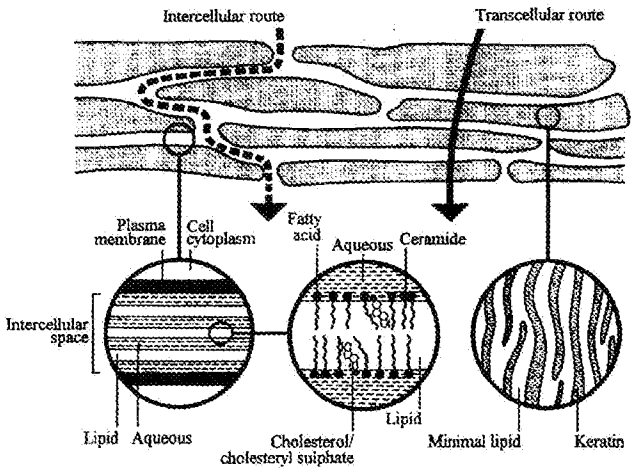

Fig. 2. Simplified diagram of stratum corneum and two microroutes of drug penetration.

although they may bind e.g. testosterone, inhibiting its systemic removal.

4. Optimising transdermal drug delivery

Fig. 3 summarises some ways for circumventing the stratum corneum barrier.

4.1. Drug and vehicle interactions

4.1.1. Selection of correct drug or prodrug

The simplest approach chooses a drug from a congeneric series or pharmacological class with the correct physicochemical properties to translocate across the barrier at an acceptable rate. A useful way to consider factors affecting drug permeation rate through stratum corneum is via the simple equation for steady state flux (Eq. (1); Barry, 1983). In general, features controlling such permeation also similarly modify short time or finite dose (depleting) situations. If we plot the cumulative mass of diffusant, m, passing per unit area through the membrane, at long times the graph approaches linearity and its slope yields the steady flux, dm/dt, (Eq. (1))

$$\frac{dm}{dt} = \frac{DC_0K}{h} \quad (1)$$

where C_0 is the constant concentration of drug in donor solution, K is the partition coefficient of solute between membrane and bathing solution, D is the diffusion coefficient and h is thickness of membrane.

From Eq. (1), we deduce the ideal properties of a molecule penetrating stratum corneum well. These are

- Low molecular mass, preferably less than 600 Da, when D tends to be high
- Adequate solubility in oil and water — so the membrane concentration gradient (the driving force for diffusion) may be high (C_0 is large). Saturated solutions (or suspensions having the same maximum thermodynamic activity) promote maximum flux in equilibrium systems.
- High but balanced (optimal) K (too large may inhibit clearance by viable tissues)
- Low melting point, correlating with good solubility as predicted by ideal solubility theory

These features explain why transdermal patches deliver adequate amounts of nicotine for effective smoking cessation therapy — this drug illustrates all these requirements.

The partition coefficient is crucially important in establishing a high initial penetrant concentration in the first stratum corneum layer. If our agent does not possess the correct physicochemical properties (usually K is too low), a suitable prodrug may have an optimal partition coeffi-

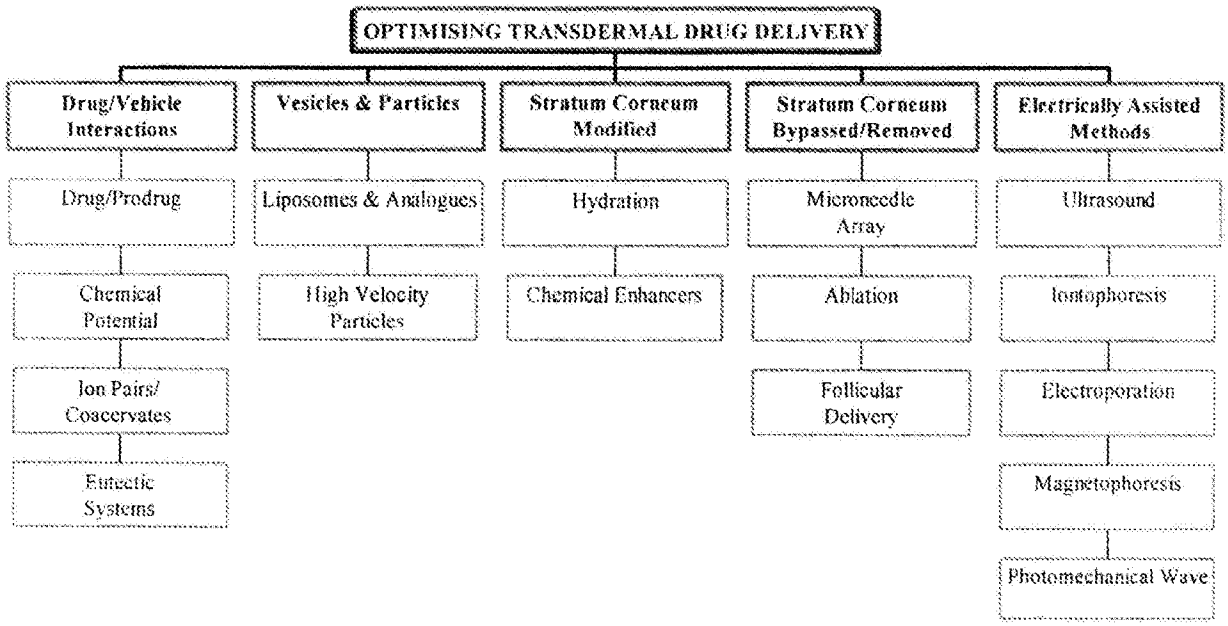

Fig. 3. Some methods for optimising transdermal drug therapy.

cient for skin entry. After permeation to viable tissues, enzymes activate the prodrug.

4.1.2. Chemical potential adjustment

An alternative form of Eq. (1) uses thermodynamic activities (Higuchi, 1960), when

$$\frac{dm}{dt} = \frac{aD}{\gamma h} \quad (2)$$

where a is the thermodynamic activity of drug in its vehicle and γ is the effective activity coefficient in the skin barrier. For maximum penetration rate, the drug should be at its highest thermodynamic activity. Now dissolved molecules in saturated solution are in equilibrium with pure solid (which by definition is at maximum activity for an equilibrated system). The solute molecules are also thus at maximum activity. Thus all vehicles containing drug as a finely ground suspension should produce the same penetration rate, provided that the systems behaves ideally i.e. D, γ and h remain constant. Ideality is difficult to maintain, as most topical vehicles interact to some extent with the horny layer.

Supersaturated solutions (i.e. nonequilibrated systems) may arise, either by design or via a cosolvent evaporating on the skin (Coldman et al., 1969). The theoretical maximum flux may then increase manyfold. Polymers may be incorporated to inhibit crystallisation in unstable supersaturated preparations. The metastability period is usually short, but may be prolonged in transdermal patches because of their mode of preparation — drug dissolution in hot solvents, evaporation to supersaturation and crystal inhibition by the polymers of the high viscosity matrix or adhesive (Kondon and Sugimoto, 1987; Kondon et al., 1987a,b; Chiang et al., 1989; Davis and Hadgraft, 1991; Kemken et al., 1992; Henmi et al., 1994; Pellett et al., 1994, 1997a,b; Fang et al., 1999; Iervolino et al., 2000; Raghavan et al., 2000; Lipp, 1998; Lipp and Muller-Fahrnow, 1999; Hadgraft, 1999). To illustrate the magnitude of the phenomenon, for estradiol at 18-times saturation, Megrab et al. (1995) achieved an 18-fold increase in stratum corneum uptake and a 13-times increase in flux.

However, Schwarb et al. (1999) were unable to show an effect of supersaturation in increasing the delivery of fluocinonide to the skin, as assessed by the vasoconstrictor assay.

4.1.3. Ion pairs and complex coacervates

Charged molecules do not readily penetrate stratum corneum. One technique forms a lipophilic ion pair, by adding an oppositely charged species. The complex partitions into the stratum corneum lipids, as charges temporarily neutralise. The ion pair diffuses to the aqueous viable epidermis, there to dissociate into its charged species, which partition into the epidermis and diffuse onward (e.g. Megwa et al., 2000a,b; Valenta et al., 2000). Stott et al. (2001) considered the relationship between ion-pair permeation of addition compounds and eutectic systems. Generally, enhancement is modest (twofold). Sometimes with penetration enhancers, it is unnecessary to consider ion-pair phenomena (Smith and Irwin, 2000).

Complex coacervation is the separation of oppositely charged ions into a coacervate oil phase, rich in ionic complex. The coacervate partitions into stratum corneum, where it behaves as ion pairs, diffusing, dissociating and

passing into viable tissues; flux enhancement is again modest (Stott et al., 1996).

4.1.4. Eutectic systems

The formulation advantages of a eutectic mixture of prilocaine and lidocaine in EMLA cream (Nyqvist-Mayer et al., 1986) prompted study of such systems for other drugs. For example, Stott et al. (1998, 2001) investigated eutectic systems of ibuprofen formed with seven terpenes and propranolol with fatty acids, correlating their interactions with increased transdermal permeation. Kang et al. (2000) showed that the lidocaine — menthol system promoted permeation through snake skin.

4.2. Vesicles and particles

4.2.1. Liposomes and other vesicles

Liposomes are colloidal particles, typically consisting of phospholipids and cholesterol, with other possible ingredients. These lipid molecules form concentric bimolecular layers that may entrap and deliver drugs to the skin. Most reports cite a localising effect whereby vesicles accumulate drugs in stratum corneum or other upper skin layers (e.g. Mezei and Gulasekharam, 1980; Mezei and Gulasekharam, 1982; Touitou et al., 1994; Fresta and Puglisi, 1996; Meidan et al., 1998a; Cheng and Chien, 1999). Generally, liposomes are not expected to penetrate into viable skin, although occasional transport processes were reported (Mezei, 1992). How well vesicles transport drugs through the skin is debatable.

This controversy grew with the introduction of transfersomes, which incorporate 'edge activators' — surfactant molecules such as sodium cholate (Planas et al., 1992; Cevc and Blume, 1992; Cevc et al., 1993, 1995, 1997; Cevc, 1996; Paul et al., 1995). The inventors claim that such vesicles, being ultradeformable (up to 10^5 times that of an unmodified liposome), squeeze through pores in stratum corneum less than one-tenth the liposome's diameter. Thus, sizes up to 200–300 nm can penetrate intact skin. Two features are claimed to be important. Transfersomes require a hydration gradient to encourage skin penetration, that is, nonoccluded conditions. Then the gradient operating from the (relatively) dry skin surface towards waterlogged viable tissues drives transfersomes through the horny layer (Fig. 4). Thus, phospholipids tend to avoid dry surroundings; a suspension of such vesicles deposited on the skin surface non-occlusively will evaporate and partially dehydrate. For vesicles to remain maximally swollen, they must follow the local hydration gradient and penetrate into more strongly hydrated and deeper skin layers of viable epidermis and dermis. Traditional liposomes in this situation are expected to confine themselves to surface or upper layers of stratum corneum, where they dehydrate and fuse with skin lipids (Cevc, 1992; Cevc and Blume, 1992; Cevc et al., 1995, 1996; Guo et al., 2000). Secondly, transfersomes work best under in vivo conditions.

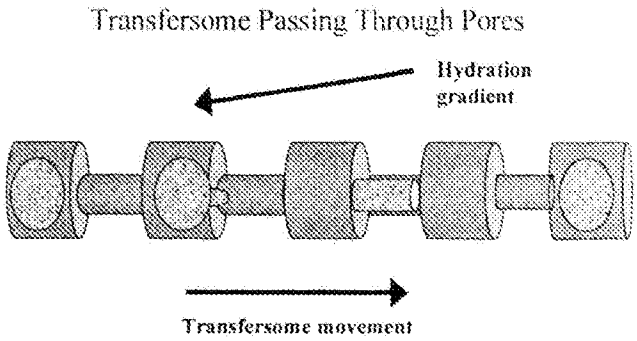

Fig. 4. Ultradeformable transfersome squeezing through minute pores in the stratum corneum, driven by the water concentration gradient. The liposome with edge-activators thus penetrates from the horny layer surface (relatively dry) to the wet viable tissues (modified from Cevc et al., 1996).

Remarkable results are claimed for transfersomes. Data indicate that as much as 50% of a topical dose of a protein or peptide (such as insulin) penetrates skin in vivo in 30 min.

Other workers measured drug delivery from ultradeformable liposomes and traditional vesicles using open and occluded conditions in vitro. Both liposome types improved maximum flux and skin deposition compared to saturated aqueous drug solution (maximum thermodynamic control) under non-occluded conditions (El Maghraby et al., 1999, 2000a,b, 2001a,b). However, positive results did not reach the values obtained by Cevc and co-workers, as only 1–3% of drug was delivered. Five potential mechanisms of action of these liposomes were assessed

1. A free drug process — drug releases from vesicle and independently permeates skin.
2. Enhancement due to release of lipids from vesicles and interaction with skin lipids.
3. Improved drug uptake by skin.
4. That different entrapment efficiencies of the liposomes controlled drug input.
5. Penetration of stratum corneum by intact liposomes.

Data indicated no evidence for (i), but revealed a possible penetration enhancing effect for pure phosphaditylcholine vesicles, although this was not the only mechanism operating. There was evidence of an uptake effect but no correlation of entrapment efficiency and drug delivery. The data did not confirm that liposomes penetrate through, as distinct from into, the horny layer, in vitro (El Maghraby et al., 1999).

Fluid liposomes delivered more fluorescein into stratum corneum than did rigid liposomes (Perez-Cullell et al., 2000).

Ethosomes are liposomes high in ethanol content (up to 45%). They penetrate skin and enhance compound delivery to deep skin strata or systemically (Touitou, 1996, 1998; Touitou et al., 2000a,b; Dayan and Touitou, 2000).

Touitou et al. (2000c) suggest that ethanol fluidises both ethosomal lipids and bilayers of the mortar (Fig. 2). The soft, malleable vesicles then penetrate through the disorganised lipid bilayers.

Niosomes use nonionic surfactants to form vesicles (Schreier and Bouwstra, 1994). Transport of entrapped spin labelled compounds into skin was examined by electron paramagnetic resonance imaging methods (Sentjurc et al., 1999) and mechanistic aspects of cyclosporin-A skin delivery were assessed by Waranuch et al. (1998). Niosomes (e.g. urea formulations Mazda et al., 1997) have been much promoted by the cosmetic industry, sometimes as almost magical ingredients.

4.2.2. High velocity particles

The PowderJect system fires solid particles (20–100 μm) through stratum corneum into lower skin layers, using a supersonic shock wave of helium gas (Burkoth et al., 1999). The claimed advantages of the system include

- Pain-free delivery — particles are too small to trigger pain receptors in skin
- Improved efficacy and bioavailability
- Targeting to a specific tissue, such as a vaccine delivered to epidermal cells
- Sustained release, or fast release
- Accurate dosing
- Overcomes needle phobia
- Safety — the device avoids skin damage or infection from needles or splashback of body fluids — particularly important for HIV and hepatitis B virus

However, there have been problems with bruising and particles bouncing off skin surfaces. Regulatory authorities will need convincing that high velocity particles smashing through the stratum corneum (Fig. 2) really do no damage to this elegant structure which is not readily repaired, nor do they carry surface contaminants such as bacteria into viable skin layers.

The leading products in development include lignocaine and levobupivacaine for local anaesthesia, proteins (follicle stimulating hormone and β-interferon) and hepatitis B DNA and other vaccines (Sarphie et al., 1997; Degano et al., 1998; Vanderzanden et al., 1998; Tacket et al., 1999; Roy et al., 2000).

The Intraject is a development of the vaccine gun designed to deliver liquids through skin without using needles. It is surprising that, after the widespread use of similar devices for vaccination — such as by the US military in Vietnam — it was not developed for drug delivery earlier.

4.3. Stratum corneum modified

4.3.1. Hydration

Hydration of stratum corneum increases the penetration rate of most (but not all) substances; water opens up the compact structure of horny layer (Menon et al., 1994). Moisturising factors, occlusive films, hydrophobic ointments and transdermal patches all enhance drug bioavailability into skin (Barry and Williams, 1995; Wester and Maibach, 1995; Haigh and Smith, 1995; Hollingsbee et al., 1965). Table 1 illustrates general effects on drug permea-

Table 1
Expected effects of skin delivery systems on horny layer hydration and skin permeability — in approximate order of decreasing hydration

Delivery system	Examples/constituents	Effect on skin hydration	Effect on skin permeability
Occlusive dressing	Plastic film, unperforated waterproof plaster	Prevents water loss; full hydration	Marked increase
Occlusive patch	Most transdermal patches	Prevents water loss; full hydration	Marked increase
Lipophilic material	Paraffins, oils, fats, waxes, fatty acids and alcohols, esters, silicones	Prevents water loss; may produce full hydration	Marked increase
Absorption base	Anhydrous lipid material plus water–oil emulsifiers	Prevents water loss; marked hydration	Marked increase
Emulsifying base	Anhydrous lipid material plus oil–water emulsifiers	Prevents water loss; marked hydration	Marked increase
Water–oil emulsion	Oily creams	Retards water loss; raised hydration	Increase
Oil–water emulsion	Aqueous creams	May donate water; slight hydration increase	Slight increase?
Humectant	Water-soluble bases, glycerol, glycols	May withdraw water; decreased hydration	Can decrease or act as penetration enhancer
Powder	Clays, organics, inorganics, 'shake' lotions	Aid water evaporation, decreased excess hydration	Little effect on stratum corneum

tion when pharmaceutical systems influence stratum corneum water content. Raised hydration may not always increase drug permeation (Bucks et al., 1989).

4.3.2. Chemical penetration enhancers

Substances temporarily diminishing the barrier of the skin, known also as accelerants or sorption promoters, can enhance drug flux. Skin enhancer literature is now so extensive that we consider only representative references, concentrating mainly on reviews.

A sample summary of enhancers includes: water, hydrocarbons, sulphoxides (especially dimethylsulphoxide) and their analogues, pyrrolidones, fatty acids, esters and alcohols, azone and its derivatives, surfactants (anionic, cationic and nonionic), amides (including urea and its derivatives), polyols, essential oils, terpenes and derivatives, oxazolidines, epidermal enzymes, polymers, lipid synthesis inhibitors, biodegradable enhancers and synergistic mixtures (Williams and Barry, 1995; Smith and Maibach, 1995 — chapters therein; Asbill and Michniak, 2000; Asbill et al., 2000; Sinha and Kaur, 2000). Reddy et al. (2000) concisely review enantioselective permeation, with and without chiral enhancers, including terpenes. The effect of ionisation and enhancers on permeation through skin and silastic has been considered (Smith and Irwin, 2000).

For safety and effectiveness, the best penetration enhancer is water (see above). Most substances penetrate better through hydrated stratum corneum than through dry tissue, hence the value of occlusive patches. Thus, any chemical which is pharmacologically inactive, non-damaging and which promotes horny layer hydration, is a penetration enhancer. Examples include the natural moisturising factor and urea.

An important theme in enhancer research is how to classify accelerant action and explain (and rationalise) the various mechanisms responsible for increased drug permeation. The hope is that by understanding fundamental principles, we move away from empirical testing of promoter activity to prediction. The structural diversity of enhancer molecules makes this a challenge.

One simple classification is via the lipid–protein–partitioning concept (Barry, 1988, 1991; Goodman and Barry, 1989; Williams and Barry, 1991a). This hypothesis suggests that accelerants act by one or more ways selected from three main possibilities (see Fig. 2). Studies by Aungst et al. (1990) broadly support this concept.

4.3.2.1. Lipid action

The enhancer disrupts stratum corneum lipid organisation, making it permeable. The essential action increases the drug's diffusion coefficient (Eq. (1)). The accelerant molecules jump into the bilayer, rotating, vibrating and translocating, forming microcavities and increasing the free volume available for drug diffusion. Without enhancer, the free volume fraction is lowest (and D lowest) near the bilayer interface. Even a slight increase in free volume fraction as enhancers molecules congregate there dramatically increases D. The bilayer centre is always somewhat disordered, with a high free volume, so that enhancer effects on diffusivity here are marginal.

Many enhancers operate mainly in this way (e.g. azone, terpenes, fatty acids, DMSO and alcohols). It was assumed that such enhancers would penetrate into, and mix homogeneously with, the lipids. However, oleic acid and terpenes, at high loadings, pool within lipid domains i.e. they phase-separate. Permeable 'pores' form which, for polar molecules, allow easier access to viable epidermis (Ongpipattanakul et al., 1991; Cornwell et al., 1994, 1996).

Some solvents (e.g. DMSO, ethanol) and micellar solutions may also extract lipids, making the horny layer more permeable through forming aqueous channels (Menczel, 1995). Menon et al. (1998) discuss well solvent effects on the lipid domain of the horny layer.

4.3.2.2. Protein modification

Ionic surfactants, decylmethylsulphoxide and DMSO interact well with keratin in corneocytes, opening up the dense protein structure, making it more permeable, and thus increasing D (Eq. (1)). However, the intracellular route is not usually important in drug permeation, although drastic reductions to this route's resistance could open up an alternative pathway. Such molecules may also modify peptide/protein material in the bilayer domain, a feature usually neglected in the literature (Barry, 1991).

4.3.2.3. Partitioning promotion

Many solvents enter stratum corneum, change its solution properties by altering the chemical environment, and thus increase partitioning of a second molecule into the horny layer (i.e. raise K in Eq. (1)). This molecule may be a drug, a coenhancer or a cosolvent (including water). For example, ethanol increases the penetration of nitroglycerine and estradiol. Propylene glycol is also widely employed, particularly to provide synergistic mixtures with molecules such as azone, oleic acid and the terpenes i.e. to raise the horny layer concentration of these enhancers.

In theory, nonsolvent enhancers that mainly act to raise drug diffusivity by mechanisms discussed above (lipid action) should also increase the partition coefficient for lipid drugs. That is, by disordering the lipid interfacial domain they increase free volume and make a larger fraction of the bilayer available for solute partitioning. The nonsolvent enhancer, of course, also affects the chemical environment throughout the lipid domain and thus, theoretically, modifies the solute partition coefficient. When only low concentrations of bilayer-disrupting agents enter the stratum corneum, we can ignore this minor effect.

Many chemical enhancers combine these three LPP mechanisms. Thus, high concentrations of DMSO (above 60%) disturb intercellular organisation, extract lipids, interact with keratin and facilitate lipid drug partitioning.

Because of the availability of extensive data on enhancer effects, investigation of structure–activity relationships is an obvious approach. Terpenes and sesquiterpenes have received this treatment (Williams and Barry, 1991; Cornwell and Barry, 1994). Other attempts (based on factors such as chain length, polarity, unsaturation and presence of special groups) were summarised by Kanikkannan et al. (2000).

An alternative scheme for classifying enhancer action uses a conceptual diagram of three areas based on the organic and inorganic characters of enhancers (Hori et al., 1989, 1990). Area I encloses enhancers which are solvents, Area II designates accelerants for hydrophilic drugs and Area III contains promoters for lipophylic compounds. Barry and Williams (1995) applied their data on terpenes to this conceptual diagram, showing that it predicted the activity of some terpenes but not others. A shortcoming of the scheme is that it implies that an enhancer may be effective for either hydrophilic or hydrophobic compounds. However, a terpene such as 1,8-cineole promotes the penetration of polar 5-fluorouracil and lipophylic estradiol. (The scope for enhancement of hydrophilic drugs is greater than that for hydrophobic penetrants. We can allow for this in comparing the activities of enhancers by using an enhancement index; see Williams and Barry, 1991b).

An unfortunate feature of many potent enhancers (which can be deduced from their abilities to disrupt organised lipid structures) is that they irritate, as they also interfere with viable cell membranes. Industrial scientists therefore often limit their investigations for a suitable enhancer to materials known to be benign on skin e.g. GRAS (generally regarded as safe) substances. For example, the insect repellent *N,N*-diethyl-*m*-toluamide is also an enhancer and is now formulated into an estradiol patch.

Mitragotri (2000) discussed synergy between chemical enhancers and electrically assisted methods of (ultrasound, iontophoresis and electroporation; see Section 4.5 and Fig. 5).

4.4. Stratum corneum bypassed or removed

4.4.1. Microneedle array

Stratum corneum can be bypassed by injection and one development of this approach is a device of 400 microneedles which insert drug just below the barrier (Henry et al., 1998; McAllister et al., 2000; Asbill et al., 2000). The solid silicon needles (coated with drug) or hollow metal needles (filled with drug solution) penetrate the horny layer without breaking it or stimulating nerves in deeper tissues; the feel to the skin is rather like a cat's tongue, or sharkskin. Flux increases up to 100 000-fold are claimed. The technique may also be combined with iontophoresis. It would be interesting to see if the microneedle approach could be combined with a microchip to control the release of the drug through the needles (Santini et al., 1999; Langer, 2000).

4.4.2. Stratum corneum ablated

As the horny layer usually provides the permeation barrier, we could consider simply removing it. Chemical peels may provide superficial or light (epidermal), medium (epidermal–dermal junction) or deep (deep papillary or papillary reticular dermis) treatments. Microdermabrasion uses a stream of aluminium oxide crystals and dermabra-

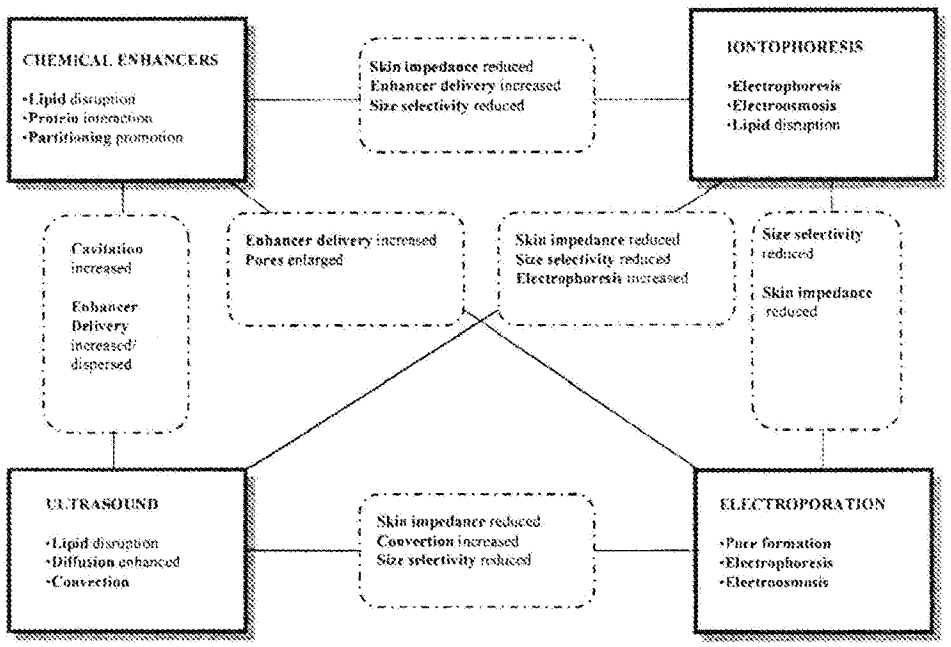

Fig. 5. Suggested mechanisms for the actions of transdermal penetration enhancers (in main rectangular boxes) and possible synergistic actions between methods as illustrated in connecting boxes (rounded rectangles). Modified from Mitragotri (2000).

sion employs a motor-driven abrasive fraise or cylinder (Friedland and Buchel, 2000). Laser ablation applies high-powered pulses to vapourise a section of the horny layer so as to produce permeable skin regions (Dover et al., 2000). The apparatus is costly and requires expert operation to avoid damage such as burns — it is inappropriate for home use.

Adhesive tape can remove stratum corneum prior to drug application; tape-stripping is used to measure drug uptake into skin (Touitou et al., 1998; Bashir et al., 2001). A microinfusor device has been proposed to delivery peptides, proteins and other macromolecules (Meehan et al., 1997). One other method forms a blister by suction, an epidermatome removes the raised tissue, after which a morphine solution delivered directly to the exposed dermis produces fast pain relief (Svedman et al., 1996).

4.4.3. Follicular delivery

The pilosebaceous unit (hair follicle, hair shaft and sebaceous gland) provides a route that bypasses intact stratum corneum; it also represents a drug delivery target. The sebaceous gland cells are more permeable than corneocytes and thus drugs can reach the dermis by entering the follicle (bypassing the invaginated stratum corneum), passing through the sebaceous gland or penetrating the epithelium of the follicular sheath. The rich blood supply aids absorption, even though the shunt route cross-sectional area is small.

Even such a large molecule as 'naked' DNA can immunise by topical application and the use of the hair follicle as a gene therapy target is exciting (e.g. Fan et al., 1999; Hoffman, 2000). It was speculated that normal follicles have efficient mechanisms for inducing immune responses to proteins in the follicle. A preparation made from antibodies from transgenic plants, when rubbed into the scalp, neutralised hair-loss effects of toxic chemicals used in chemotherapy. Colloidal particles, such as liposomes and analogues (e.g., Tschan et al., 1997 Weiner, 1998; Agarwal et al., 2000; Touitou et al., 2000) and small crystals (Allec et al., 1997) target the hair follicle. In general, particles >10 μm remain on the skin surface, those ≈ 3–10 μm concentrate in the follicle and when <3 μm, they penetrate follicles and stratum corneum alike (Schaefer and Redelmeier, 1996).

The importance of shunt route penetration of liposomes was researched using a novel technique whereby the shunts were blocked by a second layer of stratum corneum (El Maghraby et al., 2000b). Shunts played only a very minor role in liposomal delivery to lower skin layers.

4.5. Electrically assisted methods

4.5.1. Ultrasound (phonophoresis, sonophoresis)

This technique, used originally in physiotherapy and sports medicine, applies a preparation topically and massages the site with an ultrasound source. The procedure was extended to transdermal drug delivery studies (Kost, 1995; Camel, 1995; Byl, 1995; Mitragotri and Kost, 2000). The ultrasonic energy (at low frequency) disturbs the lipid packing in stratum corneum (see Fig. 2) by cavitation. Shock waves of collapsing vacuum cavities increase free volume space in bimolecular leaflets and thus enhance drug penetration into the tissue (Menon et al., 1994; Mitragotri et al., 1995a,c; Simonin, 1995; Ueda et al., 1995; Liu et al., 1998).

Initial investigations suffered from using standard high frequency devices that focused the energy into deeper, muscular tissues, rather then the correct delivery target, the stratum corneum. Now, however, low frequency (~ 20 kHz) rather than therapeutic ultrasound (~ 1 MHz upwards) increases enhancement a thousand-fold (Mitragotri et al., 1995c, 1996, 2000d). Below a threshhold value for cavitation (which depends on conditions, Langer, 2000), promotion is inversely proportional to frequency. As usual, a clear goal is the transdermal delivery of large polar molecules, and work on the phonophoresis of insulin, erythropoietin and interferon is especially significant (Mitragotri et al., 1995b; Tachibana, 1992).

Other investigations have shown: a possible deactivation of skin enzymes by ultrasound (Hikima et al., 1998); effect of pulsed delivery (Asano et al., 1997); synergistic co-operation of ultrasound with iontophoresis (Le et al., 2000), penetration enhancers (Johnson et al., 1996; Mitragotri et al., 2000a) and electroporation (Kost et al., 1996); phonophoresis used to probe the relative contribution of the follicular route to the penetration of hydrophilic permeants (Meidan et al., 1998b); and its potential for the transdermal extraction of analytes (Mitragotri et al., 2000b,c; Cantrel et al., 2000).

A problem is the need to validate the technique for effectiveness and safety in patients. As yet, it is not readily suitable for home use.

4.5.2. Iontophoresis

Iontophoresis, the electrical driving of charged molecules into tissue, passes a small direct current (approximately 0.5 mA/cm^2) through a drug-containing electrode in contact with the skin. A grounding electrode elsewhere on the body completes the circuit (Sage, 1995; Banga, 1998; Guy, 1998). Three main mechanisms enhance molecular transport: (a) charged species are driven primarily by electrical repulsion from the driving electrode; (b) the flow of electric current may increase the permeability of skin; and (c) electroosmosis may affect uncharged molecules and large polar peptides. Efficiency of transport depends mainly on polarity, valency and mobility of the charged species, as well as electrical duty cycles and formulation components (Naik et al., 2000).

Considerable interest is now shown in possible transdermal delivery of therapeutic peptides (Miller et al., 1990; Bhatia and Singh, 1998a,b; Chiang et al., 1998), proteins (Mitragotri et al., 1995c) and oligonucleotides (Oldenburg

et al., 1995; Brand et al., 1998), as well as many other drugs. Clinical trials have proceeded with lidocaine and fentanyl (Banga, 1998; Gupta et al., 1998).

Polar neutral molecules can be delivered by a current-induced convective flow of water — electroosmosis (Banga, 1998; Sims and Higuchi, 1990; Pikal, 1992; Delgado-Charro and Guy, 1994; Peck et al., 1996; Lin et al., 1997; Burnette and Ongpipattanakul, 1987; Singh et al., 1998; Merino et al., 1999; Bath et al., 2000). Thus, at above pH~4, stratum corneum is negatively charged and therefore the preferential transport of small cations such as buffer components (e.g. Na^+) imposes a net solvent flow from anode to cathode, carrying with it unionised species (or even cations). Elecroosmosis may even be the main force driving peptides and proteins through skin.

A lidocaine–epinephrine (adrenaline) device for local anaesthesia is now available, and work proceeds on the development of iontophoretic patch systems (Naik et al., 2000).

As for other enhancing techniques, workers investigate the synergy of iontophoresis with e.g. penetration enhancers (Bhatia and Singh, 1998a,b; Choi et al., 1999; Wang et al., 2000) and ultrasound (Le et al., 2000; Mitragotri et al., 2000).

An interesting development is reverse iontophoresis by which molecules in the systemic circulation (such as glucose) can be extracted at the skin surface using the electroosmotic effect (Tamada et al., 1995; Santi and Guy, 1996). The GlucoWatch Biographer aims to monitor blood glucose concentrations in diabetics using this procedure (Naik et al., 2000).

A problem with iontophoresis is that, although the apparent current density per unit area is low, most of the current penetrates via the low resistance route i.e. the appendages, particularly hair follicles (Abramson and Engle, 1941; Grimmes, 1984; Burnette and Ongpipattanakul, 1988; Cullander and Guy, 1991; Scott et al., 1993;). Thus the actual current density in the follicle may be high enough to damage growing hair. (Pores, whose identity has not been elucidated, may also contribute to iontophoretic flux — Burnette and Ongpipattanakul, 1988; Wearley et al., 1989). There is also concern about other possible irreversible changes to the skin. The biophysical effects of iontophoresis (and electroporation) have been reviewed by Jadoul et al. (1999) and Curdy et al. (2001).

As for ultrasound, there is the problem of home use, although considerable work has been done on miniaturising systems e.g. paper batteries and wristwatch-like devices are being investigated.

4.5.3. Electroporation

Skin electroporation (electropermeabilization) (Prausnitz et al., 1993) creates transient aqueous pores in the lipid bilayers (Fig. 2) by application of short (micro- to millisecond) electrical pulses of approximately 100–1000 V/cm. These pores provide pathways for drug penetration that travel straight through the horny layer (Pliquett et al., 1996; Prausnitz et al., 1996; Higuchi et al., 1999; Teissie et al., 1999; Jadoul et al., 1999; Weaver, 2000). During the pulse, drugs transport via iontophoresis and/or electroosmosis. Significant movement can also occur between pulses by simple diffusion due to relatively persistent changes in the stratum corneum lowering its resistance (Prausnitz, 1999).

Fluxes increased $10–10^4$-fold for neutral and highly charged molecules of up to 40 kDa (Vanbever and Preat, 1995, 1998; Prausnitz et al., 1995; Zewert et al., 1995; Zhang et al., 1996, 1997; Vanbever et al., 1996; Jadoul and Preat, 1997; Wang et al., 1997; Regnier et al., 1997, 1999, 2000; Lombry et al., 2000; Chang et al., 2000). The process may also transport into the integument, vaccines (Misra et al., 1999), liposomes (Badkar et al., 1999), as well as nanoparticles and microspheres (Prausnitz et al., 1996; Hofmann et al., 1995), although failures have been reported (Cheng et al., 1999). An interesting development is electroporation used to deliver physostigmine as a pretreatment for anticipated organophosphate poisoning (Rowland and Chilcott, 2000).

Macromolecules and small molecules may enhance electroporation by stabilising sterically pores created in skin (Vanbever et al., 1997; Weaver et al., 1997; Zewert et al., 1999; Ilic et al., 1999). Ilic et al. (2001) propose microengineering aqueous pathways for transdermal delivery and for sampling skin fluids.

Electroporation may combine with iontophoresis to enhance the penetration of peptides such as vasopressin, neurotensin, calcitonin and LHRH (Bommannan et al., 1994; Riviere et al., 1995; Banga et al., 1999). Electroporation has also been combined with ultrasound (Kost et al., 1996).

Again, instrumentation for home use for this potent technique is problematical, and concern relating to possible skin damage requires further study (Prausnitz, 1999; Vanbever and Preat, 1999).

Mitragotri (2000) has published an excellent thought-provoking review of synergistic interactions between chemical enhancers (Section 4.3 and Fig. 5) and ultrasound, iontophoresis or electroporation.

4.5.4. Magnetophoresis

Limited work probed the ability of magnetic fields to move diamagnetic materials through skin (Murthy, 1999). Langer (2000) discussed the interesting idea of employing intelligent systems based on magnetism or microchip technology to deliver drugs in controlled, pulsatile mode (Santini et al., 1999).

4.5.5. Photomechanical wave

A drug solution, placed on the skin and covered by a black polystyrene target, is irradiated with a laser pulse. The resultant photomechanical wave stresses the horny

layer and enhances drug delivery (Lee et al., 1999). The technique is likely to remain experimental.

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